Attorney Docket No. 5470.255

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: Xiong et al.
Application Serial No: 09/541,462
Filing Date: March 31, 2000
For: *Isolated DNA encoding cullin regulators ROC1 and ROC2, isolated proteins encoded by the same, and methods utilizing the same*

Confirmation No.: 3846
Group Art Unit: 1652
Examiner: D. Ramirez

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF YUE XIONG AND TOMOHIKO OHTA
UNDER 37 C.F.R. § 1.131

Sir:

We, YUE XIONG and TOMOHIKO OHTA, hereby declare that:

1. We are the inventors of the subject matter of the rejected claims pending in the above-referenced patent application.

2. Prior to February 26, 1999, we conceived and reduced to practice the compositions and methods recited in pending claims 1, 3-7, 13-16 and 49.

3. In support of the above statement, we enclose as Appendix A, a copy of a letter from co-inventor, Dr. Yue Xiong, to Dr. Brownen Nishikawa, a technology development associate in the Office of Technology Development at the University of North Carolina at Chapel Hill, explaining that a manuscript is enclosed with this letter that describes the discovery and functional characterization of two novel human genes, ROC1 and ROC2. A copy of the manuscript referenced in the letter is also enclosed as Appendix B. All dates on these documents have been blocked out, but are before February 26, 1999.

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4.   Specifically, the manuscript enclosed with the above-described letter is a draft of the manuscript which formed the basis of the present specification and that was subsequently published (Ohta et al. "ROC1, a homolog of APC11, represents a family of culllin partners with an associated ubiquitin ligase activity" *Molecular Cell* 3:535-541 (April, 1999). As indicated in the figure legend on page 34 and in the attached figures, the nucleotide sequence and amino acid sequence of the ROC1 protein disclosed in the manuscript is identical to the nucleotide sequence and amino acid sequence of the ROC1 protein disclosed in the present application as SEQ ID NOs:1 and 2, respectively.

5.   In summary, our statements herein and the documents concurrently submitted show conception and reduction to practice of the claimed invention prior to February 26, 1999.

7.   We hereby declare that all statements made herein of our own knowledge are true, and that all statements made on information and belief are believed to be true. We further declare that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Yue Xiong	11/22/04
	Date
Tomohiko Ohta	
	Date

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so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the
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application or any patent issued thereon.

Yue Xiong	Date
Tomohiko Ohta	11/29/2005
	Date

UNC Lineberger Comprehensive Cancer Center

SCHOOL OF MEDICINE
CAMPUS BOX 7295
UNIVERSITY OF NORTH CAROLINA AT CHAPEL HILL
CHAPEL HILL, NC 27899-7295
(919) 966-3036   Telefax (919) 966-3015

Tel: (919)-962-2142
Fax: (919)-966-8799
Email: yxiong@email.unc.edu

Bronwen Nishikawa, Ph.D.
Technology Development Associate
Office of Technology Development
CB # 4105
308 Bynum Hall
University of North Carolina at Chapel Hill
Chapel Hill, NC 27599-4105

APPENDIX A

Dear Bronwen:

Please find enclose manuscript by Ohta et al., on the discovery and functional characterization of two novel human genes, ROC1 (previously named as CUD1) and ROC2 (previously named as CUD2), that we previously disclosed to your office. This manuscript was submitted to <u>Cell</u> for consideration of publication on

As you will find from the manuscript, ROC1 and ROC2 genes represent a new family of cullin regulators. Cullins form potentially a large number of ubiquitin ligases to control degradation of different proteins. The exact function of cullins has just began to be revealed (the first gene cullin gene was described in 1996), but is believed to be critical for many diverse cellular processes, ranging from cell proliferation, cell death, transcription, signal transduction, antigen presentation, tumor suppressor etc. ROC1 and ROC2 is the first proteins identified as critical subunits and general regulators of cullins. and thus have the potential to regulate wide range of cellular processes as well. As such, I believe that these two new genes will have significant potential for many pharmaceutical companies engaged in the discovery of drugs related to different diseases. Therefore, I urge you and your office to expedite the process to file protection of these two genes.

Sincerely,

Yue Xiong, Ph.D.
Assistant Professor
Biochemistry and Biophysics
Member, Lineberger Comprehensive Cancer Center

ROC1, a homologue of APC11, represents a candidate family of cullin ubiquitin ligase regulators

Tomohiko Ohta (1, 7), Jennifer J. Michel (2), Peilin Tan (6), Zhen-Qiang Pan (6) and Yue Xiong (1-5)

(1) Lineberger Comprehensive Cancer Center, (2) Curriculum in Genetics and Molecular Biology, (3) Department of Biochemistry and Biophysics, (4) Program in Molecular Biology and Biotechnology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-3280. (6) Mount Sinai School of Medicine, New York, NY 10029

(5)To whom correspondence should be addressed at: yxiong@email.unc.edu

(7) Permanent address: First Department of Surgery, St. Marianna University School of Medicine, Kawasaki, 216 Japan;

Summary

Cullins belong to a family of evolutionarily conserved proteins that potentially form a large number of E3-like ubiquitin ligases. We have identified two closely related novel RING finger proteins, ROC1 and ROC2, that are homologous to APC11, a subunit of the APC ubiquitin ligase that contains a cullin-related component, APC2. ROC1 and ROC2 commonly interact with all cullins, while APC11 specifically interacts with APC2. The expression of *ROC1, 2* and *APC11* genes is induced following mitogenic stimulation. Unlike cullins and other cullin associated proteins, ROC1, 2 and APC11 are short lived proteins, and the turnover of ROC1 is governed by the proteasome pathway. The ROC1 protein promotes nuclear import of cullin proteins. Co-expression of CUL1 and ROC1, but not either alone, results in high levels of ubiquitin ligase activity. Hence, ROC and APC11 proteins may function as regulators of cullin and APC ubiquitin ligases during interphase and mitosis, respectively.

Introduction

Most short lived intracellular proteins whose concentrations change promptly as the result of alterations in cellular physiological conditions are regulated by the ubiquitin-dependent proteolytic process (Hochstrasser, 1996; King et al., 1996; Hershko, 1997). This process, in addition to performing such housekeeping functions as homeostasis and removing misfolded proteins, is involved in degrading many regulatory proteins, such as cyclins, CDK inhibitors, transcription factors, and signal transducers. In brief, ubiquitin-mediated proteolysis begins with activation of ubiquitin, a 76 amino acid protein expressed in all eukaryotic cells, in an ATP dependent manner by a ubiquitin activating enzyme (E1 or Uba). The charged ubiquitin forms a high energy thiolester bond with E1 and is passed to a cysteine residue within an ubiquitin-conjugating enzyme designated as an E2 or Ubc. E2-linked ubiquitin is then transferred to a side chain amino group of a lysine residue in the substrate to form a terminal isopeptide bond, either directly or often indirectly targeted by a ubiquitin ligase known as E3. This series of reactions results in the accumulation of proteins covalently conjugated with polyubiquitin chains which are rapidly detected and degraded by the 26S proteasome. E3, functionally defined as an activity that is both necessary and sufficient for transfer of ubiquitin from a ubiquitin-charged E2 to a substrate, is believed to provide substrate specificity and may contain multiple subunits. Because most polyubiquitinated proteins are indiscriminately delivered to the 26S proteasome for degradation, elucidating the mechanism and regulation of E3 ligases activity have become critical issues central to our understanding of regulated proteolysis.

Knowledge about E3 ubiquitin ligases is very limited at present. Among the few characterized E3 ligases are the N-end rule ubiquitin ligase E3α/Ubr1 that recognize proteins by binding to the basic or hydrophobic residues at the amino-termini of substrate proteins [reviewed in (Varshavsky, 1996)]; the HECT (homologous to E6-AP carboxy terminus) domain proteins represented by mammalian E6-AP which functions as a ubiquitin-ligase for p53 (Scheffner et al., 1993; Huibregtse et al., 1995; Scheffner et al., 1995); and the APC (anaphase-promoting complex or

cyclosome), a 20S complex that consists of 8 to 12 subunits and is required for both entry into anaphase as well as exit from mitosis [(King et al., 1995; Sudakin et al., 1995; Zachariae et al., 1998; H. Yu et al. 1998), reviewed in (King et al., 1996)]. The APC plays a crucial role in regulating the passage of cells through anaphase by promoting ubiquitin-dependent proteolysis of many proteins. In addition to destructing the mitotic B-type cyclin for inactivation of CDC2 kinase activity and initiating cytokinesis [reviewed in (Hochstrasser, 1996; King et al., 1996; Hershko, 1997)], the APC is also required for degradation of other proteins for sister chromatid separation and spindle disassembly, including the anaphase inhibitors PDS1 (Cohen-Fix et al., 1996) and CUT2 (Funabiki et al., 1996), ASE1 (Juang et al., 1997) and the cohesion protein SCC1P (Michaelis et al., 1997). All known proteins degraded by the APC contain a conserved nine amino acid stretch commonly known as the destruction box that is necessary for their ubiquitination and subsequent degradation (Glotzer et al., 1991). Proteins that are degraded during G1, ranging from G1 cyclins and CDK inhibitors to transcription factors, do not contain the conserved destruction box or any other common structural motif. Instead, substrate phosphorylation appears to play an important role in targeting their interaction with E3 for subsequent ubiquitination. Genetic and biochemical analysis has identified in yeast an E3-like activity, dubbed as the SCF, that plays a key role in regulating G1 progression. The SCF consists of at least three subunits, SKP1, CDC53/cullin and an F-box containing protein, in which SKP1 functions as an adaptor to connect CDC53 to the F-box protein which binds directly to the substrate (Bai et al., 1996; Willems et al., 1996; Verma et al., 1997; Skowyra et al., 1997; Feldman et al., 1997).

In a screen for mutants with excess postembryonic cell divisions in C.elegans, a gene, cullin-1, was identified whose loss of function caused hyperplasia of all tissues as a result of the failure to properly exit from the cell cycle (Kipreos et al., 1996). CUL1 represents an evolutionarily conserved multigene family that includes at least seven members in C.elegans, six in humans, and three in budding yeast (Kipreos et al., 1996; Mathias et al., 1996), and contains significant sequence homology to yeast Cdc53p (Mathias et al., 1996). Like yeast CDC53, human cullin 1

directly binds to SKP1 to form a multi-subunit complex with SKP2 (an F box protein), cyclin A and CDK2 (Lisztwan et al., 1998; Michel and Xiong, 1998; Lyapina et al., 1998; Z. K. Yu et al. 1998), and can assemble into functional, chimeric ubiquitin ligase complexes with yeast SCF components (Lyapina et al., 1998), purporting the cullins as components of ubiquitin ligase complexes. Very recently, a subunit of the mitotic APC E3 complex, APC2, was found to contain limited sequence similarity to CDC53/cullins (Zachariae et al., 1998; H. Yu et al. 1998), reinforcing the notion that cullin proteins function as E3 ubiquitin ligases.

The regulation of E3 ligases is poorly understood. The activity of the APC is cell cycle regulated, active from anaphase until late G1 (Amon et al., 1994; King et al., 1995; Brandeis and Hunt, 1996). The principle regulation is probably provided by subunit rearrangements such as CDC20 and CDH1 binding (Visintin et al., 1997; Schwab et al., 1997; Sigrist and Lehner, 1997; Fang et al., 1998), and phosphorylation of certain subunits may also play an important, but supplementary role (Lahav-Baratz et al., 1995; King et al., 1995; Peters et al., 1996). Regulation of CDC53 and cullin-mediated E3 ligase activity during interphase is yet to be determined. The steady state levels of both CUL1, SKP1 and the CUL1-SKP1 complex remain constant throughout the cell cycle and persist in postmitotic cells (Michel and Xiong, 1998), suggesting that if the activity of a CUL1-dependent ubiquitin ligase changes in response to mitogen or oscillates during the cell cycle, it is regulated by mechanisms other than the abundance of CUL1 or SKP1. We report here the identification of two closely related RING finger proteins, ROC1 and ROC2, that share a high degree of sequence similarity with APC11, a subunit of the APC complex. We present evidence consistent with the hypothesis that ROC and APC11 proteins function as candidate regulators of cullin and APC ubiquitin ligases during interphase and mitosis, respectively.

Results

ROC1 interacts directly with all cullins

In a yeast two-hybrid screen for cellular proteins that could interact with the cullin family of proteins, we screened a human HeLa cDNA library using the mouse cullin 4A as a bait. Full length mouse CUL4A encodes a 759 amino acid protein and shares 96% identity with human CUL4A that was recently identified as candidate 13q amplicon target gene and was amplified or overexpressed in high percentage of breast cancer samples (Chen et al., 1998). An estimated 3 x 10^6 transformants were screened. Of 17 clones isolated from this screen that grew on histidine deficient selective medium, 11 corresponded to the gene, named ROC1(regulator of cullins), as determined by DNA sequencing and diagnostic restriction digestion analysis. In addition to CUL4A, ROC1 can also interact with cullin 1, 2 and 5 as determined by the yeast two-hybrid assay (Figure 1A). Cullin 3 which interacts with ROC1 very weakly in yeast cells was later found to also bind to ROC1 in cultured mammalian cells (see below). Thus, ROC1, unlike SKP1 which selectively interacts with CUL1 only [(Michel and Xiong, 1998), Figure 1A], may be a general cullin-interacting protein.

The mammalian cullin genes encode a family of closely related proteins with molecular weights of approximately 90 kDa. CUL1 interacts with SKP1 via an NH_2-terminal domain (Michel and Xiong, 1998). To determine the structural basis underlying the specific interaction between cullins and ROC1, we mapped the region of CUL1 required for its interaction with ROC1. A series of CUL1 deletions from both amino- and carboxyl- terminals fused in-frame with the yeast *Gal4* DNA binding domain were tested for their abilities to interact with ROC1 in yeast cells. ROC1 interacts with the C-terminal 527 amino acid residues of CUL1, but not the N-terminal 249 residues of CUL1 (Figure 1B). In contrast, SKP1 binds to the N-terminal domain of CUL1. These results indicate that CUL1 contains at least two distinct domains, an N-terminal domain for interacting with SKP1 and a C-terminal domain for binding with ROC1. Such structural separation suggests that ROC1 is unlikely interacting with CUL1 in a competing manner

with SKP1. Hence, ROC1 and SKP1 may co-exist in the same protein complex with CUL1 to perform different functions.

ROC1 represents a family of RING finger proteins related to APC11

ROC1 encodes an 108 amino acid residue protein with a predicted molecular weight of 12265 D (Figure 1C). Database searches identified ROC1 as a highly evolutionarily conserved gene whose *S.cerevisiae* (ROC1-Sc), *S.pombe* (ROC1-Sp) and plant (ROC1-At) homologues share 67%, 88% and remarkably 98% protein sequence identity with human ROC1, respectively, over the 82 amino acid region compared (Figure 1E). Database searches have also identified two additional genes, ROC2 in higher eukaryotes (Figure 1D) and APC11 in all eukaryotic species (Figure 1E), that are closely related to ROC1. Human ROC2 and APC11 encode an 85 amino acid (Mr. 10007 D) and an 84 residue (Mr. 9805 D) protein, respectively. ROC1 and ROC2 share an overall protein sequence identity of 51% with each other and 38% and 35% identity with APC11, respectively, indicating that ROC1 and ROC2 are more closely related to each other than to APC11. Like ROC1, both ROC2 and APC11 are also highly conserved during evolution. Therefore, ROC1/ROC2/APC11 define a new family of proteins that are likely to carry out important cellular functions.

ROC/APC11 proteins contain two characteristic features: a RING finger and richness in tryptophan residues. The RING finger domain has been found in many eukaryotic proteins with diverse functions and is thought to mediate protein-protein interactions (Borden and Freemont, 1996). The majority of RING finger proteins contain a highly conserved structural motif with a histidine residue flanked by three and four cysteine residues on either side (C_3HC_4). Notably, the ROC1 protein from all species has a substitution of the last cysteine with an aspartic acid residue (Figure 1E). The second feature of this family of proteins is six highly conserved tryptophan residues. Three tryptophan residues in ROC1 are followed by an acidic amino acid residue (Asn,

Glu or Asp) that resemble the WD repeat and may potentially also be involved in mediating protein-protein interactions.

APC11 was recently identified as a subunit of the yeast APC complex whose loss of function resulted in a defect in the onset of anaphase and exit from mitosis (Zachariae et al., 1998). Another APC subunit, APC2, was found to contain limited sequence similarity to the C-terminal region of cullins (Zachariae et al., 1998; H. Yu et al. 1998). These observations, together with our finding that both ROC1 and ROC2 (see below) directly interact with cullins, suggest 1) that APC11 may directly interact with APC2, 2) that the region for interacting with ROC and APC11 may be located to the conserved C-terminal portion in cullins and APC2 proteins, and 3) that ROC proteins may function in regulating ubiquitin-dependent proteolysis.

In vivo association of ROC1 and cullins

To confirm the interaction between ROC1 and cullin proteins, Saos-2 cells were transfected with plasmids directing the expression of HA-epitope tagged human ROC1 (HA-ROC1) together with CUL1 or other individual myc-epitope tagged cullins. Transfected cells were metabolically labeled with [^{35}S]-methionine, and cell lysates were immunoprecipitated reciprocally with either anti-HA, anti-CUL1 or anti-myc antibody (Figure 2A). Neither the myc antibody cross-reacted with ROC1 (e.g. lanes 2 and 3, Figure 2A) nor the HA antibody cross-reacted with the cullins (lane 12, Figure 2A, and also lanes 6 - 9 of Figure 3D). All five cullins were co-precipitated with ROC1 by the HA antibody. In the reciprocal immunoprecipitations, HA-ROC1 protein was detected readily in anti-myc-mCUL4A by the myc antibody, but was not evident in anti-myc-cullin 2, 3 and 5 immunocomplexes. The difference in the reciprocal immunoprecipitations is not clear, but may be related to the efficiency of the anti-myc antibody in precipitating other myc tagged cullins. Un-tagged CUL1 formed a complex with co-transfected ROC1 with equal efficiency as myc tagged cullins (lane 1), excluding the possibility of any artifactual binding between ROC1 and cullin proteins that might be caused by myc epitope tagging or cross-reactivity of the myc antibody. In

addition to the ROC1-cullin association, several cellular proteins of unknown identity were precipitated with either ROC1 or a cullin protein, including an 110 kDa cellular protein (p110) that was co-precipitated with HA-ROC1 when CUL1, but not other cullins, was co-expressed (Figure 2A, lane 7).

To further confirm the ROC1-cullins association, total protein lysates were prepared from Saos-2 cells transfected with plasmids directing the expression of HA-ROC1 and CUL1 or individual myc-epitope tagged cullins. Lysates were sequentially immunoprecipitated and immunoblotted (IP-Western) using antibodies against HA, myc or CUL1 (Figure 2B). Cullin 1, 2, 4A and 5 were readily detected in the anti-HA immunocomplex. Consistent with the yeast two-hybrid assay, cullin 3 was not seen in the ROC1 complex as determined by IP-Western (lane 4), suggesting that ROC1 may only interact weakly with cullin 3.

To obtain evidence for in vivo ROC1-cullin association under more physiological conditions, we raised a rabbit polyclonal antibody specific to ROC1. This antibody is capable of precipitating both ROC1 and the ROC1-CUL1 complex as determined by the use of in vitro translated proteins (lanes 1 and 2, Figure 2C). From metabolically labeled HeLa and Saos-2 cells, the anti-ROC1 antibody precipitated a protein of approximately 15 kda (lanes 3 and 5). This 15 kda protein apparently corresponds to ROC1, as judged by its co-migration with in vitro produced ROC1 and by competition using the antigen peptide (lanes 4 and 6). In addition to ROC1, a number of cellular proteins between 75 to 200 kda were coprecipitated with ROC1. The presence of these proteins in the anti-ROC1 immunocomplex is blocked by the competing antigen peptide, suggesting that these proteins may specifically associate with ROC1. This observation is consistent with the finding that ROC1 is broadly interacting with all cullin proteins. To demonstrate the in vivo ROC1-cullins association without overexpression, HeLa cell lysate was immunoprecipitated with the anti-ROC1 antibody, and precipitates were analyzed by IP-Western using antibodies specific to either cullin 1 or cullin 2. As shown in Figure 2D, both cullins were

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readily detected in the ROC1 immunocomplexes and were specifically blocked by the competing antigen peptide. Demonstration of association between ROC1 and other cullins was not carried out because of the lack of antibodies to other cullins at present. Unfortunately, this anti-ROC1 antibody is unable to recognize denatured ROC1 protein immobilized on the nitrocellulose, preventing us from performing reciprocal IP-Western analysis and determining the steady state level of ROC1 protein in un-transfected cells.

Selective interaction between ROC2, APC11 and cullin family proteins

We next determined whether ROC2 and APC11, like ROC1, also interact with cullins using the yeast two-hybrid assay and an in vivo binding assay. Full length human ROC2 or APC11 were fused in-frame with the yeast *Gal4* DNA activation domain and co-transformed into yeast cells with individual cullins fused to the *Gal4* DNA binding domain. Almost identical to ROC1, ROC2 interacted strongly with cullins 1, 2, 4A and 5 (Figure 3A), indicating that ROC2 is also a general cullin-interacting protein. In contrast, APC11 only interacted with cullin 5, but not other cullins (Figure 3B).

To further test the interaction between ROC2 and APC11 with cullins, Saos-2 cells were transfected with plasmids directing the expression of HA tagged human ROC2 (HA-ROC2) or APC11 (HA-APC11) together with untagged CUL1 or individual myc tagged cullins. Transfected cells were metabolically labeled with [^{35}S]-methionine, and cell lysates were immunoprecipitated with either anti-HA, anti-CUL1 or anti-myc antibody (Figures 3C and 3D). Transfected HA-ROC2 protein migrates as a doublet (lanes 6 to 10, Figure 3C). Whether endogenous ROC2 also expresses two forms and the nature of these two forms remain to be determined. The myc antibody does not cross-react with either form of ROC2 (e.g. comparing lanes 5 and 6). All five cullins were co-precipitated with ROC2 by the HA antibody (lanes 6 to 10). Reciprocally, ROC2, preferentially the faster migrating form, was also detected in cullin 2, 3 and 4 immunocomplexes (lanes 2 to 4).

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In contrast and with the exception of cullin 5, APC11 and cullins were not detected to interact with each other in reciprocal precipitations (lane 1 to 10, Figure 3D). Cullin 5 was weakly, but reproducibly, detected in the APC11 immunocomplex (lane 10). Of all six mammalian cullins, CUL5 is the most divergent member of the cullin family and contains the highest sequence similarity to APC2. Whether CUL5 uniquely has a role in APC function, as implicated by its interaction with APC11, remains to be determined. In addition to the cullins, several cellular proteins including a band of approximately 110 kDa was detected in the ROC2 complex when CUL5, but not other cullins, was co-expressed (Figure 3C, lanes 5 and 10). Notably, p110 was not detected in cells co-transfected with CUL5 and ROC1 (lane 11 of Figure 2A) or APC11(lane 10 of Figure 3D). Whether this ROC2-CUL5-associated p110 is related to the ROC1-CUL1-associated p110 (Figure 2A, lane 7) and the functional roles these proteins may play in cullin-ROC complexes have not been determined. We also noticed that cullin 2, 3 and 4 immunocomplexes, when precipitated from cells co-transfected with ROC2, but not APC11, contained a cellular protein of approximately 17 kda. The presence of this 17 kda polypeptide was not evident in either CUL1 or CUL5 immunocomplexes which contained little ROC2, suggesting the possibility that its association with cullin 2-4 is correlated with, and may actually dependent on or is promoted by, the association of cullins with ROC2.

Specific interaction between APC11 and APC2

APC11 was co-purified with another APC subunit, APC2, which contains limited sequence similarity to cullins (Zachariae et al., 1998; H. Yu et al. 1998), leading us to test whether APC11 directly interacts with APC2. When tested by the two-hybrid assay, APC11, but not ROC1 nor ROC2, interacted with mouse APC2 in yeast cells (Figure 4A). To assess the interaction between APC2 and these three closely related RING finger proteins in mammalian cells, we transfected HeLa cells with plasmids directing the expression of myc-epitope tagged APC2 with either HA-epitope tagged ROC1, ROC2 or APC11. Transfected cells were metabolically labeled with [^{35}S]-

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methionine and cell lysates were immunoprecipitated reciprocally with either anti-HA or anti-myc antibody (Figure 4B). Consistent with the yeast two-hybrid assay, APC2 was seen in the APC11 immunocomplex (lane 4), and reciprocally, APC11 was detected in the APC2 immunocomplex (lane 9), indicating that within the APC complex (which consists of at least 12 subunits), APC11 is directly binding with APC2. Ectopically expressed ROC1 was seen to weakly interact with APC2 in mammalian cells (lane 2 and 7), raising the intriguing possibility that ROC1 may have a function in APC activity. This observation, however, must be interpreted cautiously, as lack of immunological reagents to APC2 and other APC subunits at present prevented us from determining whether ROC1 interacts with APC under more physiological conditions. Consistent with the yeast two-hybrid assay, ROC2, even when overproduced, was not seen to interact with APC2 (lanes 3 and 8), indicating that ROC2 specifically interacts with cullins, but not APC2.

Expression of *ROC1, ROC2 and APC11* genes are induced during G1

To test whether ROC/APC11 genes might carry out a regulatory role in cullin and APC complexes, we first examined the expression of these genes during cell cycle transition (Figure 5A). Both ROC1 and ROC2 mRNAs, as well as CUL1 mRNA, are expressed at a readily detectable levels in quiescent and G1 cells, become evidently induced at the G1-to-S transition following mitogenic stimulation, and remain relatively constant throughout the cell cycle. These observations suggest that if CUL1- and other cullin-associated activity is inactivated in quiescent cells and/or oscillates during the cell cycle, the steady state level of ROC1 and ROC2 mRNA is unlikely to play a significant role to regulate such change. APC11 mRNA, on the other hand, was nearly undetectable in quiescent cells and is induced following mitogenic stimulation, suggesting that one potential mechanism of turning off the APC activity in quiescent cells may be the inhibition of APC11 gene expression.

ROC1, ROC2 and APC11 are short lived proteins

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We determined the half-life of transfected HA-ROC1 (~26 minutes), HA-ROC2 (~9 minutes) and HA-APC11 (~17 minutes) by pulse-chase experiments and found that all three proteins are very short lived (Figure 5B). In contrast, transfected CUL1 and endogenous SKP1 have estimated half lives of 2.5 and 2 hours, respectively (Figure 5B). We also determined the half-life of another CUL1 interacting protein, the E2 CDC34. The half-life of transfected CDC34 was longer than the 5 hours of our experimental time course (Figure 5B), and the endogenous CDC34 was not even evidently detected after 4 hours of pulse labeling despite the abundant amount of CDC34 protein expressed as determined by direct immunoblotting (data not shown). This feature, together with their mitogen-induced expression pattern during the cell cycle, suggests that ROC1, ROC2 and APC11 may act as rate-limiting factors of the function of the cullin-associated ubiquitin ligase activities.

Turnover of ROC1 protein is sensitive to proteasome inhibitors

The short half-lives of ROC and APC11 proteins led us to determine if their turnover is regulated by ubiquitin-mediated proteolysis. HeLa cells transfected with HA-ROC1 expressing plasmid were treated with LLnL (N-acetyl-leucinyl-leucinyl-norleucinal) or MG132 (carbobenzoxyl-leucinyl-leucinyl-leucinal), both inhibitors of 26S proteasome activity. Treatment of transfected cells with either proteasome inhibitor, but not solvent DMSO (dimethylsulfoxide), increased the half life of ROC1 protein to around 90 minutes (Figure 5C), indicating that ROC1 degradation is regulated through an LLnL- and MG132-sensitive pathway, likely by the ubiquitin-mediated 26S proteasome pathway.

ROC1 promotes nuclear import of cullin proteins

Cullin proteins localize predominantly to the cytoplasm [(Pause et al., 1997), (Figure 6C, 6D, 6K and 6L)], whereas many substrate proteins of ubiquitin ligases are in the nucleus, suggesting the need for a regulatory mechanism for either exporting cullin substrate proteins or importing cullin ubiquitin ligase activity. Unlike cullins, ROC1 protein, when overexpressed by transient

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transfection, localized mainly to the nucleus and fairly to the cytoplasm (Figure 6A and 6B). Co-transfection with ROC1 led to a nuclear accumulation of all five cullin proteins in a significant portion of cells (Table 1). When co-transfected with ROC1, CUL1 remained in cytoplasm in only 20% of transfected cells. Of remaining 80% of transfected cells, half exhibited both cytoplasm and nuclear staining of CUL1, and the other half displayed almost exclusive nuclear staining of CUL1 (Table 1). A representative field of CUL1-ROC1 co-transfected cells showing both cytoplasmic and nuclear staining of CUL1 is shown in Figure 6 (Figure 6F). It is unclear whether the heterogeneity of CUL1 localization when co-expressed with ROC1 is related to different cell cycle phases or to the different ratio between the two proteins. Consistent with the binding studies, co-transfection of CUL1 with APC11 which exhibited similar cytoplasm and nuclear staining as ROC1, had no detectable effect on CUL1's localization (Table 1, and Figure 6H - 6J). Similar to CUL1, transfected mCUL4A (Table 1, and Figure 6K and 6N) and two other cullins, CUL2 and CUL3 (data not shown), were localized predominantly to the cytoplasm, and were localized to the nucleus in a significant portion of cells by co-transfection with ROC1. Distinctively, CUL5, when singly transfected, displayed a cytoplasmic or cytoplasmic-nuclear localization in equal portions of transfected cells, and became predominately nuclear localized when co-transfected with ROC1 (Table 1). Very few CUL5 and ROC1 double positive cells had cytoplasmic CUL5 staining. The basis for such a difference is not known at present, but seems in accordance with the distinction of CUL5 in the cullin family, including its sequence divergence and interaction with APC11. These findings suggest that one function of ROC1 may be to promote nuclear import of cullins and that targeting nuclear proteins to ubiquitin ligases may involve nuclear import of ubiquitin ligase activity.

ROC1 is a critical subunit of cullin ubiquitin ligase activity

CDC53, the closest yeast homologue of human CUL1, assembles into a functional E3 ubiquitin ligase complex in insect cells with E2 CDC34, SKP1 and an F box protein (SCF complex) to catalyze ubiquitination of phosphorylated substrates (Skowyra et al., 1997; Feldman et al., 1997).

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Protein complexes containing human CUL1, SKP1 and SKP2 assembled in insect cells, however, were found to contain little ubiquitin ligase activity, but became active after incubating with HeLa cell lysate (Lyapina et al., 1998), raising the possibility that an additional rate limiting component(s) is required for cullin-dependent ubiquitin ligase activity. To determine whether ROC1 may function as a key subunit of cullin ligase activity, we analyzed the ubiquitin ligation activity catalyzed by the ROC1/CUL1 immunocomplex isolated from 293T cells transiently transfected with DNA expressing the recombinant proteins (the details of this transfection-based CUL1 ubiquitin ligase activity assay will be described elsewhere, Z.-Q. Pan, in preparation). The ROC1- and CUL1-associated ubiquitin ligation activity was measured by incubating the ROC1/CUL1 immunocomplex immobilized on protein A agarose beads with purified human E1, mouse E2 CDC34, ATP and ^{32}P-labeled ubiquitin (Ub). After incubation, the reactions were terminated by boiling the samples in the presence of SDS and reducing agent (0.1 M DTT) and mixtures were resolved by SDS-PAGE, followed by autoradiography. An evident, time-course dependent ubiquitin ligation, as visualized by the incorporation of ^{32}P-Ub into covalently linked high molecular weight smear characteristic of ubiquitinated proteins, was detected when both E1 and E2 CDC34 were added to the HA-ROC1/CUL1/SKP2 immunocomplexes (lane 1, lanes 4 to 9, Figure 7A), but not when either E1 (lane 2) or E2 (lane 3) was omitted, indicating an E1 and E2 dependent-ubiquitin ligation. As a control, anti-HA precipitate derived from cells transfected without a HA-tagged protein exhibited only E1- or E2-linked mono-ubiquitin conjugates (lanes 5 and 8, Figure 7B). The observed protein ladder reflects an increment of a single ^{32}P-Ub (~12 kDa in the form of a recombinant protein), a characteristic of ubiquitination reaction. The treatment of the reaction mixture with DTT, SDS and boiling significantly reduced, but cannot completely abolish the Ub-E1 (marked as ^{32}P-Ub-E1, Figure 7) and Ub-CDC34 (marked as ^{32}P-Ub-CDC34) conjugates.

To determine the contribution of individual proteins to the ubiquitin ligase activity in the ROC1 immunocomplex, we performed a series of "drop-out" transfections. Omission of SKP2,

an F-box protein that presumably brings substrate protein(s) to CUL1, slightly reduced the ubiquitin ligase activity (comparing lanes 2 and 3, Figure 7B). Such a non-essential role of transfected SKP2 to the ubiquitin ligase activity of the ROC1 complex may be due at least in part to the high level of endogenous SKP2 in 293 cells (Zhang et al., 1995). Omission of CUL1, however, severely reduced the ubiquitin ligase activity of ROC1 immunocomplex (lane 4), indicating the dependency of ROC1-associated ubiquitin ligase activity upon CUL1. Reciprocally, anti-HA immunocomplexes derived from cells co-transfected with HA-CUL1, myc-SKP2 and ROC1-myc, after incubation with E1, E2 and 32P-Ub, contained high levels of ubiquitin ligase activity (lane 7, Figure 7B). Omission of ROC1 from the CUL1 complex, like the omission of CUL1 from ROC1 complex, significantly reduced ubiquitin ligase activity (lane 6). Low levels of activity in the CUL1 immunocomplex without co-transfection with ROC1 may be attributed to the endogenous ROC1 protein. These observations suggest that ROC1 and CUL1 act as an integral part of a E3 ubiquitin ligase. It is critical important to point out that we do not know the identity of protein substrates that were polyubiquitinated by the CUL1 or ROC1 immunocomplex. Specific substrate of cullins has only been identified thus far for yeast CDC53. Although cyclin A is associated with CUL1 via SKP1 and SKP2, we have so far failed to detect its ubiquitination or half life change by the CUL1. Clearly, further characterization of ROC1 regulation of cullin ubiquitin ligase activity will require the identification of physiological substrates of cullins. Activation of CUL1 ligase activity by ROC1 should provide a sensitive assay to facilitate the identification of such substrates, not only for CUL1, but also for other cullins as well.

Discussion

The levels of diverse cellular proteins are subject to control by ubiquitin-mediated proteolysis resulting in the need for a large number of E3 ubiquitin ligases to target their ubiquitination. Two issues vital to our understanding of this regulated proteolysis are how the substrate targeting specificity is determined and how E3 ligase activity is regulated. The cullin family of proteins potentially form a large number of distinct E3s, as indicated by the existence of a multi-gene family (at least seven in C.elegans, and six in mammals) and by the assembly of yeast CDC53 into at least three distinct E3 complexes: with SKP1-CDC4, with SKP1-GRR1 and likely with SKP1-MET30 to mediate the ubiquitination of SIC1, CLN and SWE1 proteins, respectively (Skowyra et al., 1997; Feldman et al., 1997; Kaiser et al., 1998). It is clear that the different cullins may function in a variety of diverse cellular processes. CDC53 is required for S phase entry (Mathias et al., 1996), for coupling glucose sensing to gene expression and cell cycle (Li and Johnston, 1997), and possibly for activating mitotic CLB-CDC28 activity (Kaiser et al., 1998), the C.elegans *cul-1* mutant displays a hyperplasia phenotype (Kipreos et al., 1996), human CUL2 is associated with the tumor suppressor VHL (von Hippel-Lindau) implicated in the regulation of hypoxia-induced mRNA stability (Pause et al., 1997; Lonergan et al., 1998), human CUL4A is genomically amplified or is overexpressed in breast cancers (Chen et al., 1998), and a deficiency in the cullin-related APC2 results in mitotic arrest (Zachariae et al., 1998; H. Yu et al. 1998). In this report we describe a novel family of RING finger proteins that have the potential to commonly regulate this diversified cullin gene family. Belonging to this newly identified family are two novel proteins, ROC1 and ROC2, and a recently identified protein, APC11, a subunit of the APC E3 ligase complex (Zachariae et al., 1998; H. Yu et al. 1998). We demonstrate that ROC1 and ROC2 are commonly associated with all cullins, and we present evidence consistent with the hypothesis that the ROC/APC11 family of proteins function to regulate cullin and APC ubiquitin ligase activities.

ROC/APC11 proteins are common subunits of cullin/APC2 complexes. Both ROC1 and ROC2 interact directly with all five mammalian cullins that we have examined, as determined by

several different assays both in vitro and in vivo (Figures 1 - 3). Conventional biochemical purification has identified ROC1 as a stoichiometrically associated subunit of CUL1 ubiquitin ligase activity (Z-Q. Pan, in preparation). This provides support for the idea that ROC1 is an inherent subunit of cullin complexes as opposed to an auxiliary factor. The APC ubiquitin ligase contains a parallel association between a cullin-related protein, APC2, and a ROC homologous protein, APC11 (Figure 4), further underscoring the generality of the ROC-cullin association. These observations lead us to suggest that cullin complexes contain a core consisting of two proteins: a cullin member and a ROC member.

Several observations support the hypothesis that ROC/APC11 family proteins function as essential subunits of cullin ubiquitin ligases. First, a ROC family member, APC11, has been shown to be an essential subunit for APC ubiquitination activity. Loss of APC11 function resulted in accumulation of APC substrates and caused metaphase arrest (Zachariae et al., 1998). Similarly, omission of ROC1 dramatically reduced ubiquitin ligase activity from the CUL1 immunocomplex (Figure 7). Furthermore, we have found that interference of yeast ROC1 conformation by epitope tagging caused retarded growth with multiple, elongated buds (J.M., unpublished observation), a characteristic of cells that contain temperature-sensitive mutations in either CDC4, CDC34 or CDC53 (Mathias et al., 1996). Mutation in the RING finger region of yeast ROC1 created a dominant mutant causing nuclear segregation defects (named as YOL133W / Hrt1p, J. A. Claypool et al., in preparation). While reconstitution of CDC53- or CUL1-dependent ubiquitination activity has been reported using recombinant components without adding ROC1 (Skowyra et al., 1997; Feldman et al., 1997; Lyapina et al., 1998), we believe that the endogenous ROC proteins present in insect or mammalian cells may have compensated for the missing ROC1 in those assays. Likewise, absence of APC11 from the APC complex purified from Xenopus more likely reflects that it was missed rather than it has a non-essential function (H. Yu et al. 1998).

We present evidence arguing that the ROC/APC11 family of proteins function to regulate cullin/APC2 activity. First, the expression of all three genes of this family are low in quiescent cells and are induced following mitogenic stimulation (Figure 5). Generally, the rate of both protein synthesis and turnover in quiescent cells are lower than in actively dividing cells. Reduced levels of ROC and APC11 could potentially provide a mechanism for down-regulating the activity of various E3 activities, including APC activity. Next, ROC1 promotes nuclear import of cullins. While singly transfected cullins localize predominantly in the cytoplasm, co-expression of ROC1 with any of the five cullins that we have examined resulted in nuclear accumulation of the cullins in a significant portion of cells (Table 1). In contrast, cullin expression had no effect on ROC1 localization. Lastly, all three members of this family, ROC1, ROC2 and APC11, are short lived proteins. As the result of their short half-lives, they may act as rate-limiting factors in cullin complex formation, providing an irreversible and rapid deactivation mechanism of cullin ubiquitin ligases and allowing recycling of other stable subunits such as cullins, E2s and the F-box adaptor SKP1. In contrast to the ROC family, the *CUL1* gene is moderately expressed in quiescent cells and encodes a much more stable protein, making cullins less likely than ROC proteins to act as the rate-limiting factors of cullin-associated ubiquitin ligase activities. The short half lives of ROC/APC11 family proteins are intriguing in the light of their qualities as intrinsic subunits of cullin ubiquitin ligase activity. The turnover of ROC1 is regulated by an LLnL and MG132-sensitive pathway (Figure 5B and 5C), most likely by the ubiquitin proteasome pathway. It is particularly appealing to speculate the possibility that the ROC/APC11 proteins are triggered by an external signal(s) for auto-ubiquitination by their associated cullin/APC ubiquitin ligase, thereby serving as an auto-regulatory target for destroying the activity of their associated ligases.

The exact function of ROC family proteins in regulating cullin ubiquitin ligase activity is not obvious at present. One potential function of the ROC/APC11 family is to promote nuclear import of cullins. Two other cullin interacting proteins, p45^{SKP2} (Lisztwan et al., 1998) and VHL (Pause et al., 1997), have also been found to promote cullin nuclear import. It is not clear whether

ROC1 localizes in the nucleus and imports cullins autonomously, or collaborates with other proteins such as SKP2 and VHL to promote cullin import. We do not know at present whether importing cullins is the only or even a major function of ROC family proteins. Nor do we have any evidence that nuclear import is necessary for cullin function. The ubiquitous association of ROC proteins with cullins suggests that any other potential functions carried out by ROC family proteins must be common to all cullins, however, it may be distinct between cullins and APC2 as they selectively interact with ROC and APC11, respectively. We postulate that one candidate function may be bridging the E2s to the cullins. Such speculation is consistent with two observations. First, the APC appears to preferentially utilize a specific E2, E2-C (Aristarkhov et al., 1996), whereas CDC53/CUL1 specifically interacts with a different E2, CDC34 (Mathias et al., 1996; Skowyra et al., 1997; Feldman et al., 1997; Lisztwan et al., 1998; Lyapina et al., 1998). Second, deletion analysis has mapped the E2 CDC34 binding domain to the central part in CDC53 (Patton et al., 1998). The ROC1 binding domain has also been mapped to the portion of human CUL1 that includes central and C-terminal sequences (Figure 1B). One function of ROC/APC11 that we feel we can rule out is that of contributing to substrate recognition like the SKP1 protein. SKP1 functions in substrate targeting by serving as an adaptor to connect CDC53/CUL1 with a substrate-binding F-box protein (Bai et al., 1996; Skowyra et al., 1997; Feldman et al., 1997). Unlike ROC/APC11, SKP1 interacts exclusively with CUL1 [(Michel and Xiong, 1998), and Figure 1A], it binds to a CUL1 domain distinct from ROC/APC11 (Figure 1), it remains high in postmitotic cells (Michel and Xiong, 1998), and it is relatively stable (Figure 5B). These fundamental differences imply distinct functions between SKP1 and ROCs: SKP1 is involved in targeting substrate and ROCs in regulating cullins.

The most extensive studies on ubiquitin-mediated proteolysis have centered on the mitotic phase of the cell cycle and have identified the APC as the single major E3 ubiquitin ligase required to degrade most mitotic regulatory proteins. Recently, yeast CDC53 has been identified as a major E3 ligase activity regulating S phase entry. Though the in vivo function of most cullins are yet to

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be determined, some may well perform other functions unrelated to cell cycle control. Of the two E3 ligases involved in regulating cell cycle transitions, a similarity can be drawn such that each contains a cullin member and a ROC member: one contains APC11 and APC2, the other involves CUL1 and ROC1. This and the data presented in this study lead us to propose that ROC and APC11 proteins may function as regulators of cullin and APC ubiquitin ligases during interphase and mitosis, respectively. Furthermore, what is most striking is that two distinct ROC proteins exist in higher eukaryotes, both capable of directly interacting with all members of the cullin family. Whatever their exact mechanism may be in regulating cullin ubiquitin ligase activity, their combinatorial interactions with different cullins point to an enormous regulatory capacity by ROC proteins, perhaps reflecting the complexity of interphase regulation.

Experimental Procedures

cDNA clones, plasmids constructs and yeast two hybrid assay.

cDNA sequence encoding full length mouse cullin 4A was used as a bait to screen a HeLa cell derived cDNA library for cullin-interacting proteins by the yeast two-hybrid assay (Michel and Xiong, 1998). The full length cDNA clones for both human ROC2 and APC11 were isolated by PCR amplification from a HeLa cDNA library and confirmed by DNA sequencing. To identify cDNA clones encoding the full length mammalian APC2, we searched the EST database. Full length cDNA clones were not available for human APC2 in the current EST database. Instead, a near full length mouse APC2 EST cDNA clone (W13204) was identified that predicts a 823 amino acid open reading frame with a calculated molecular weight of 94 kDa. This mouse cDNA clone is one amino acid residue longer than the published human APC2 (H. Yu et al. 1998), but is missing the initiation methionine codon. Given the extremely close relatedness between mouse and human APC2 proteins (93% identity over the entire 823 residues), we used the mouse APC2 when testing for the interaction with human APC11.

For expression in mammalian cells, individual cDNA clones were subcloned into the pcDNA3 vector under the control of CMV promoter (Invitrogen), pcDNA3-HA or pcDNA3-Myc, for expressing HA or myc epitope tagged fusion protein. For the yeast two-hybrid assay, individual cullin sequences were cloned into pGBT8, a modified version of pGBT9, in frame with the DNA-binding domain of *Gal4*. ROC1, ROC2 and APC11 were cloned into pGAD in-frame with the DNA activation domain of *Gal4*. Yeast two-hybrid expression plasmids for human CUL1, CUL1 deletion mutants and SKP1 were previously described (Michel and Xiong, 1998).

Cell Lines, culture conditions and cell transfection.

All mammalian cells were cultured in DMEM, supplemented with 10% FBS in a 37°C incubator with 5% CO_2, which include HeLa (human cervix epithelioid carcinoma), Saos-2 (osteosarcoma), U2OS (osteosarcoma), 293T (human transformed primary embryonal kidney cells), and NHF1

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(normal human foreskin fibroblast). Cell transfections were carried out using the LipofectAMINE reagent according to the manufacturer's instructions (Gibco-BRL). For each transfection, 2 or 4 μg of total plasmid DNA (adjusted with pcDNA3 vector DNAs) were used for each 36 mm or 60 mm dish.

Synchronization of NHF1 cells was achieved by serum starvation and stimulation. NHF1 cells were cultured in DMEM containing 15% FBS to a 30-40% density, starved in DMEM containing 0.5% FBS for 3 days and stimulated by switching to DMEM containing 15% FBS. Progression through the cell cycle was monitored by flow cytometry analysis as previously described (Michel and Xiong, 1998). Procedures for isolation of total RNA and Northern hybridization have been described before (Li et al., 1994).

Antibodies and immunochemistry procedures

Procedures for [^{35}S]-methionine metabolic labeling, immunoprecipitation and immunoblotting have been described previously (Jenkins and Xiong, 1995). The sequence of synthetic peptides used in generating rabbit polyclonal antibodies are as follows: anti-human ROC1 (CMAAAMDVDTPSGTN, amino acid residues 1-14), anti-human CUL2 [CRSQASADEYSYVA, residues 733-745, (Kipreos et al., 1996; Michel and Xiong, 1998)]. A cysteine (underlined) was added to the N-terminus of each peptide for covalent coupling of the peptide to activated keyhole limpet haemocyanin (KLH). Antibodies to human CUL1 and SKP1 were previously described (Michel and Xiong, 1998). All rabbit polyclonal antibodies used in this study were affinity purified using respective peptide columns following the manufacturer's instruction (Sulfolink Kit, Pierce, Rockford, IL). Monoclonal anti-HA (12CA5, Boehringer-Mannheim), anti-myc (9E10, NeoMarker), rabbit polyclonal anti-HA antibody (Santa Cruz), rhodamine- and fluorescein (FITC)-conjugated goat anti-rabbit and anti-mouse IgG (Jackson ImmunoResearch laboratory)

antibodies were purchased commercially. Coupled in vitro transcription and translation reactions were performed using the TNT kit following the manufacturer's instructions (Promega).

Pulse-chase experiment

1×10^5 cells were seeded onto a 6-well dish and were transfected after overnight culture with a total of 2 µg of appropriate plasmid DNA and incubated for 24 hours. Cells were pulse labeled with [^{35}S]-methionine for 30 minutes, washed twice with pre-warmed 1X PBS and chased by culturing in DMEM/10% FBS media for the time indicated in each figure. To determine the regulation of protein turnover by proteasome, LLnL (final concentration of 100 µM), MG132 (final concentration of 50 µM) or the same volume of DMSO solvent (5 µl/ml culture) were added to the transfected cells during the last three hours of transfection and during pulse-chase labeling period. Lysates from pulse-chase labeled cells were immunoprecipitated with antibodies as indicated in each figure.

Indirect immunofluorescence

U2OS cells were seeded onto a 6-well plate and transfected 24 hours later with appropriate plasmid DNA (2 ug / well). All subsequent steps were carried out at room temperature. Twenty-four hours after transfection, cells were washed three times with PBS and fixed in PBS containing 3% formaldehyde for 15 minutes. After fixation the cells were washed once with PBS and permeabilized in cold PBS containing 0.2 % Triton X-100 for 5 minutes. The cells were incubated with PBS containing 0.5% BSA as a blocking agent for 30 minutes followed by incubation with the blocking buffer containing primary antibody (affinity-purified polyclonal anti-CUL1 antiserum at a final concentration of 2 µg/ml, anti-HA monoclonal antibody at 1:30 dilution of 12CA5 hybridoma culture supernatant, anti-myc monoclonal antibody at 1:30 dilution of 9E10 hybridoma culture supernatant, or affinity-purified polyclonal anti-HA antiserum at a final concentration of 2 µg/ml) for 1 hour. The cells were then incubated with 4 µg/ml of either FITC-conjugated affinity-purified goat anti-rabbit IgG and rhodamine-conjugated affinity-purified goat anti-mouse IgG, or

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FITC-conjugated affinity-purified goat anti-mouse IgG and rhodamine-conjugated affinity-purified goat anti-rabbit IgG for 30 minutes. Between different antibody incubations, cells were washed three times with PBS. Stained cells were covered by fluorescent mounting medium (DAKO) and examined with an Olympus IX70 microscope fitted with appropriate fluorescence filters.

Ubiquitin ligase activity assay

The detailed procedures for the purification of human E1 and mouse E2 CDC34, the preparation of ^{32}P-labeled ubiquitin, as well as immuno-purification of the ROC1/CUL1 containing E3 ligase complex from transiently transfected 293T cells (Figure 7) will be described elsewhere (Z-Q. Pan, in preparation). The immunopurified ROC1/CUL1 containing complex immobilized on protein A agarose beads was added to an ubiquitin ligation reaction mixture (30 µl) that contained 50 mM Tris-HCl, pH7.4, 5 mM MgCl$_2$, 2 mM NaF, 10 nM Okadaic Acid, 2 mM ATP, 0.6 mM DTT, 1 µg ^{32}P-Ub, 60 ng E1 and 300 ng mouse CDC34. The incubation was at 37^{0}C for 60 min unless otherwise specified. The reaction mixture was then added to 20 µl 4X Laemmli loading buffer and boiled for 3 min prior to 7.5% SDS-PAGE analysis.

Acknowledgments

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Figure legend

Figure 1 ROC1 interacts with members of the cullin family

(A) Yeast HF7c cells were co-transformed with plasmids expressing indicated proteins (key) and plated onto media lacking leucine and tryptophan (-LW) to verify the expression of both bait (Leu+) and prey (Trp+) plasmids; or onto media lacking leucine, tryptophan and histidine (-LWH) to assay for interactions between bait and prey proteins.

(B) ROC1 interacts with the C-terminal portion of CUL1. HF7c yeast cells were co-transformed with plasmids expressing indicated proteins. Protein-protein interaction was assayed as described in Figure 1A.

(C) Nucleotide and amino acid sequences of human ROC1. The stop codon is indicated by an asterisk.

(D) Nucleotide and amino acid sequences of human ROC2. The stop codon is indicated by an asterisk.

(E) Sequence Comparison of ROC/APC11 family of proteins from representative organisms: human (Hs, Homo sapiens), fruit fly (Dm: Drosophila melanogaster), nematodes (Ce: Caenorhabditis elegans), mouse ear cress (At: Arabidopsis thaliana), fission yeast (Sp: Schizosaccharomyces pombe), and budding yeast (Sc: Saccharomyces cerevisiae). Only residues that are identical to all sequences are in bold. The number in the bracket of certain sequences indicates the length of insertion omitted. The number preceding and following each sequence indicates the position of the first amino acid residue in each gene and the total length of each protein, respectively.

Figure 2 In vivo association of ROC1 with cullins

(A) [35S]-methionine labeled lysates were prepared from HeLa cells transfected with plasmids expressing the indicated proteins. Lysates were divided into two equal amounts and immunoprecipitated with indicated antibodies and resolved by SDS-PAGE.

34

(B) Total cell lysates were prepared from HeLa cells transfected with plasmids expressing indicated proteins. Lysates were immunoprecipitated with anti-HA monoclonal antibody. After SDS-PAGE, proteins were transferred to nitrocellulose, and various portions of the same blot were analyzed by Western analysis using antibodies to CUL1 (lanes 1 and 2, top), myc (lanes 3 to 6, top), and HA (lanes 1 to 6, bottom).

(C) [^{35}S]-methionine labeled, in vitro translated ROC1 (lane 1), mixture of ROC1 and CUL1 (lane 2), or cell lysates from HeLa and Saos-2 cells were immunoprecipitated with anti-ROC1 antibody with (+) or without (-) pre-incubation of the competing ROC1 antigen peptide as indicated at the top of each lane. After several washings, precipitates were resolved by SDS-PAGE.

(D) Total cell lysates prepared from HeLa cells were immunoprecipitated with indicated antibodies. After SDS-PAGE, proteins were transferred to nitrocellulose, and analyzed by Western analysis using antibodies to CUL1 (lanes 1 to 4) or to CUL2 (lanes 5 to 8).

Figure 3   Interaction between ROC2, APC11 and cullin family proteins

(A and B) HF7c yeast cells were co-transformed with plasmids expressing human ROC2 or human APC11 and various cullins. pGBT8-PCNA and pGAD vector plasmid were included as negative controls. Protein-protein interactions were determined by the yeast two-hybrid assay as described in Figure 1A.

(C and D) Interaction between ROC2, APC11 and cullin family proteins in mammalian cells. HA-tagged ROC2 or APC11 were co-transfected with vectors expressing CUL1 or myc-tagged individual cullin proteins into HeLa cells. Two days after transfection, cells were pulse labeled for 2 hours with [^{35}S]-methionine. Cell lysates prepared from the labeled cells were divided into two equal amounts, immunoprecipitated with the indicated antibodies and resolved by SDS-PAGE. All five cullin proteins were co-precipitated with HA-ROC2, but only CUL-5 co-precipitated with APC11.

35

Figure 4 Interaction between ROC1, ROC2, APC11 and APC2

(A) Selective interaction between APC2 and ROC or APC11. HF7c yeast cells were co-transformed with plasmids expressing indicated proteins (key). Protein-protein interaction was determined by the yeast two-hybrid assay using selective medium lacking histidine (-LWH) supplemented with 5 mM 3-AT to suppress the low trans-activating activity of GAL4^{BD}-APC2 fusion protein ("self-activation").

(B) [^{35}S]-methionine labeled lysates were prepared from HeLa cells transfected with plasmids expressing indicated proteins. Lysates were divided in half, immunoprecipitated with indicated antibodies and resolved by SDS-PAGE.

Figure 5. Cell cycle expression of *ROC1, ROC2* and *APC11* mRNA and protein half-lives

(A) Normal human foreskin diploid fibroblasts (strain NHF1) were arrested in G0 by serum deprivation and released from quiescence by serum stimulation. Total RNA was prepared from cells at different time points after stimulation by serum readdition. 5 ug of RNA from each preparation were loaded on a 1.5% agarose gel, and equal RNA loading was further confirmed by ethidium bromide staining (lower panel). Resolved RNA was transferred to a nitrocellulose filter, and the blot was hybridized with a series of probes derived from the coding region of indicated human cDNAs. As an alternative control for monitoring the cell cycle progression, the same blot was also hybridized with a probe corresponding to the G1 accumulated CDK inhibitor p21 (Li et al., 1994).

(B) ROC1 is a short lived protein. 24 hours after Saos-2 cells were transfected with vectors expressing individual proteins (except SKP1 which was measured for endogenous protein), cells were pulsed with [^{35}S]-methonine for 30 minutes and then chased for the indicated length of time. Cell lysates were precipitated with anti-HA (for ROC1, ROC2 and APC11), anti-CUL1, anti-CDC34 and anti-SKP1 antibodies, resolved by SDS-PAGE and autoradiographed. The amount of each labeled protein at each time point was quantitated on a PhosphoImager, normalized relative to the amount of radiolabeled protein in cells chased for 0 hr, and plotted against the chase time.

(C) Stabilization of ROC1 protein by proteasome inhibitors. HeLa cells were transfected with a plasmid expressing HA tagged ROC1 and 24 hours later were treated with LLnL, MG132 or control solvent DMSO for 3 hours. Cells were then pulsed with [^{35}S]-methionine for 30 minutes and then chased for the indicated length of time. Cell lysates were precipitated with anti-HA antibodies, resolved by SDS-PAGE and autoradiographed. The amount of each labeled protein at each time point was quantitated on a PhosphoImager, normalized relative to the amount of radiolabeled protein in cells chased for 0 hr, and plotted against the chase time.

Figure 6   ROC1 localizes cullins to the nucleus.

Subcellular localization of ROC1, APC11   (red fluorescence), CUL1 and mCUL4A (green fluorescence) were examined by indirect fluorescence staining. U2OS cells were transiently transfected with HA-ROC1 (A and B), CUL1 (C and D), HA-ROC1 and CUL1 (E - G), HA-APC11 and CUL1 (H - J), myc-CUL4 (K and L), and HA-ROC1 and myc-mCUL4A (M - O). 24 hours after transfection, cells were fixed and stained with mouse anti-HA antibody (A, E and H) and rabbit anti-CUL1 antibody (C, F and I) followed by rhodamine-conjugated (red fluorescence) anti-mouse and FITC-conjugated (green fluorescence) anti-rabbit antibody. The phase contrast of each photographed field are shown (B, D, G, J, L and O). To examine myc-mCUL4A in cells co-transfected with HA-ROC1 (K, M and N), a rabbit polyclonal anti-HA antibody and a mouse anti-myc antibody were used with rhodamine-conjugated anti-rabbit antibody and FITC-conjugated anti-mouse antibody. Both HA and myc antibodies exhibited no cross reactivity to cellular proteins, as indicated by the negative staining of untransfected cells in the field.

Figure 7   ROC1 stimulates cullin-dependent ubiquitin ligase activity

(A) Lysates from human 293T cells transiently transfected with plasmids expressing indicated proteins were mixed with protein A beads linked to anti-HA antibodies. HA-immunocomplexes immobilized on the beads were washed and then mixed with purified E1, E2 CDC34 (unless
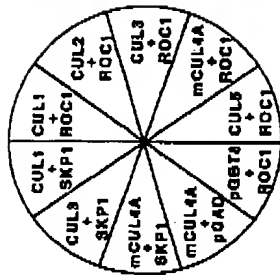
otherwise indicated), ^{32}P-labeled ubiquitin (Ub) and ATP. After 60 minutes incubation (unless otherwise specified), the reactions were terminated by boiling the samples in the presence of SDS and DTT and mixtures were resolved by SDS-PAGE, followed by autoradiography.

(B) Ubiquitin ligase activity was assayed as in (A) using lysates (~ 1 mg of total proteins) derived from cells transfected with plasmids expressing different combination of proteins as indicated.
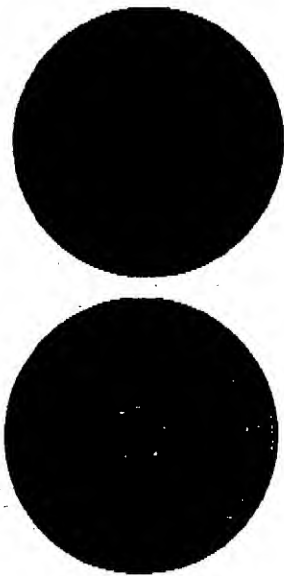
Table 1 Cellular localization of transfected cullins and ROC1 proteins

Indirect immunofluorescence staining following transfections of indicated plasmids was performed as described in Figure 6. One hundred positive cells from each transfection were microscopically examined. Localization of overexpressed proteins was defined as cytoplasmic (as shown in Figure 6C, 6I and 6K), both nuclear and cytoplasmic (as shown in Figure 6F, left side), or mainly nuclear (as shown in Figure 6A, 6E, 6F, 6H, 6M and 6N), and was expressed as a percentage of the total transfected population. The data represent the mean and standard deviation of five independent experiments except for myc-mCUL4A and myc-CUL5 which represent one experiment.
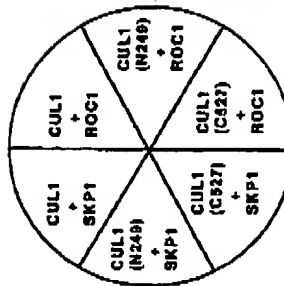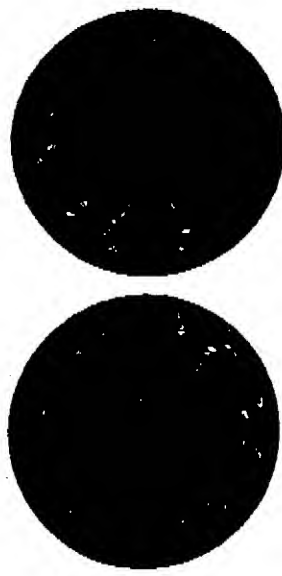
A

Key

CUL1 + SKP1 | CUL1 + ROC1 | CUL2 + ROC1
CUL3 + SKP1 | | CUL3 + ROC1
mCUL4A + SKP1 | mCUL4A + ROC1 | mCUL4A + ROC1
mCUL4B + ROC1 | pGBT9 + pGAD | CUL5 + ROC1

— LW   — LWH

B

Key

CUL1 + SKP1 | CUL1 + ROC1
CUL1 (N249) + SKP1 | CUL1 (N249) + ROC1
CUL1 (C527) + SKP1 | CUL1 (C527) + ROC1

— LW   — LWH

C

N249   C527

C *ROC1*

```
ATGGCGCAGCGATGGATGTGGATACCCGAGCGGCGGCAAACAGCGGCGGCGGGCAGGCGCTTTGAAGTGAAAAAAGTGAATGCAGTA    90
 M  A  A  M  D  V  D  T  P  S  G  T  N  S  G  A  G  K  K  R  F  E  V  K  K  W  N  A  V      30
 ·
GCCCTCTGGGCCTGGGATATTGTGGTTGATAACTGTGCCATCTGCAGGAACCACATTATGGATCTTTGCATAGAATGTCAAGCTAACCAG    180
 A  L  W  A  W  D  I  V  V  D  N  C  A  I  C  R  N  H  I  M  D  L  C  I  E  C  Q  A  N  Q    60
 ·
GCGTCCGCTACTTCAGAAGAGTGTACTGTCGCCATGGGGAGTCTGTAACCATGCCTTTCACTTCCACTCGCATCTCTCGCTGGCTCAAAACA    270
 A  S  A  T  S  E  E  C  T  V  A  W  G  V  C  N  H  A  F  H  F  H  C  I  S  R  W  L  K  T     90
 ·
CGACAGGTGTGTCCCATTGGACAACACAGAGAGTGGGAATTCCAAAAGTATGGGCACTAG    327
 R  Q  V  C  P  L  D  N  R  E  W  E  F  Q  K  Y  G  H  *     108
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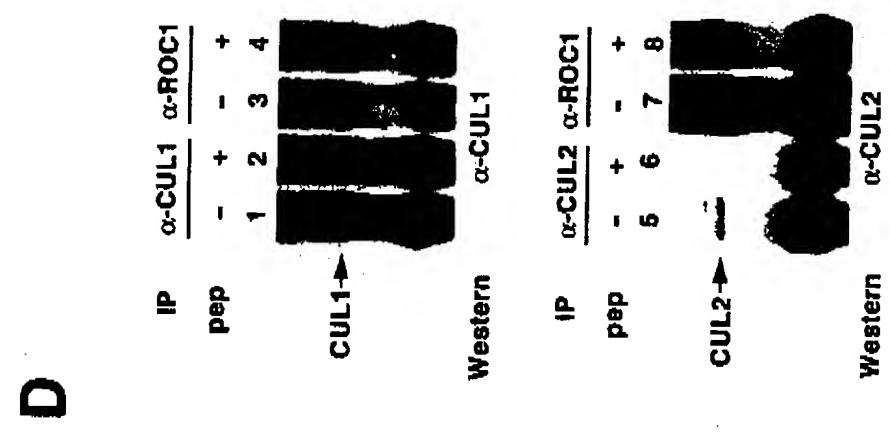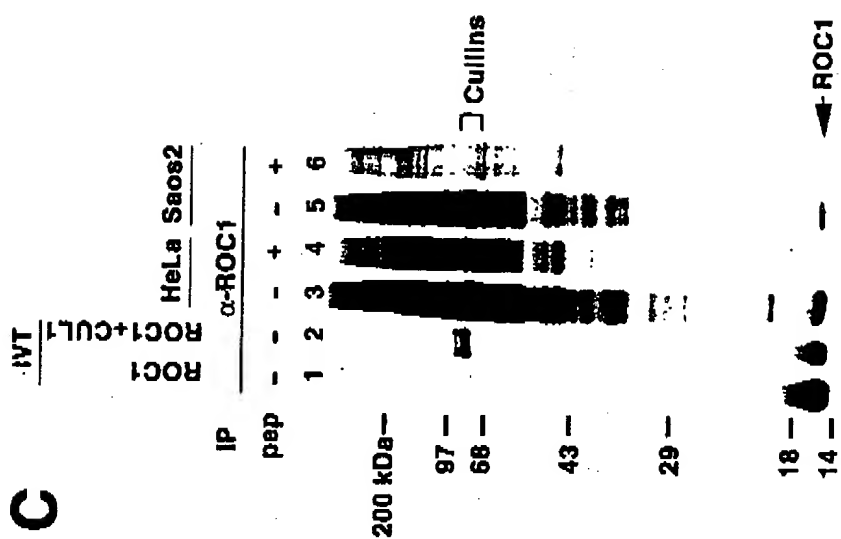
D *ROC2*

```
ATGTTCTCCCTCAAGAAGTGGAACCCCGGTGCCATGTGGAGCTGGGACGTCGAGTGCGCCATCTGCAGGGTCCAGGTGATG    90
 M  F  S  L  K  K  W  N  P  V  A  M  W  S  W  D  V  E  C  D  T  C  A  I  C  R  V  Q  V  M    30
 ·
GATGCCCTGTCTTAGATGTCAAGCTGCAGAAATGAGGACTGTGTTGTGTGGTCTGGGAGAAATGTAATCATTCCTTCCACAACTGCTGC    180
 D  A  C  L  R  C  Q  A  E  N  K  Q  E  D  C  V  V  W  G  C  N  H  S  F  H  N  C  C     60
 ·
ATGTCCCCTGTGGGTGAAAACAGAACAATCGCTGCCCTCTCTGCCAGCAGGGGTGGTCCCAAAGAATCGGCAAATGA    258
 M  S  L  W  V  K  Q  N  N  R  C  P  L  C  Q  Q  D  W  V  V  Q  R  I  G  K  *     85
```
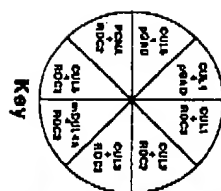
E

```
ROC1-Hs   22  FEVKKWNAVALMAMDIVVDNCAICRNHIMDLCIECQANQASATSEECTVAWGVCNHAFHFHCISRML---KTRQVCPLDNREWEF  108
ROC1-Dm   22  FEVKKWNAVALNAMDIVVDNCAICRNHIMDLCIECQANQASATSEECTVAWGVCNHAFHFHCISRML---KTRQVCPLDNREYDF  108
ROC1-Ce   24  FEVKKWSAVALNAMDIQVDNCAICRNHIMDLCIECQANQAAGLKDECTVAWGNCNHEAFHFHCISRML---KTRQVCPLDNREWEF  108
ROC1-At   32  FEIKKWNAVALNAMDIVVDNCAICRNHIMDLCIECQANQASATSEECTVAWGVCNHEAFHFHCISRML---KTRQVCPLDNSEWEF  110
ROC1-Sp   21  FBIKKWNAVALRQMDIVVDNCAICRNHIMDLCIECQANTDSAAAQECTVAWGTCNHAFHFHCISRML---NTRNVCPLDNREWEF  118
ROC1-Sc   35  FEIKKWTAVAPMSMDIAVDNCAICRNHIMRPCIECQPKAMTDTDNECVAAMGVCNHAPHLHCINKMI---KTRDACPLDNQPNQL  113
                                                                                                      121

ROC2-Hs    2  FSLKKWNAVAMMSMDVECDTCAICRVQVMDACLRCQAEN---KQEDCVVWGCNHSFHNCCMSLMV---KQNNRCPLCQQDWVV  85
ROC2-Ce   30  FVLKKWNALAVWAMDVECDTCAICRVHLMRRCLRCQSEP---SAE-CYVWGDCNHSFHHCCMTQWI---RQNNRCPLCQKDWVV  112

APC11-Hs   3  VKIKCWNGVATMLKVANDENCGICRMAFNGCCPDCKVPG-----DDCPLVWGQCSHCPHMHCILRMLHAQQVQQHCPMCRQEWKF  84
APC11-Dm   3  VTIKSWTGVATMRKIANDENCGICRMSFPBSTCPBCALPG----DDCPLVWGVCSHCPHMCIVRMLNLQPLNKQCPMCRQSWKF  85
APC11-Ce  51  ITVKKLHVCGBWKML[3]DTCGICRMEFBSACNMCKFPG-----DDCPIVLGICRHAFHRHCIDKWI[5]QPRAQCPLCRQDWTI  135
APC11-Sc   3  VKINEVHSVFAWSM[21]DVCGICRASYNGTCPSCKFPG-----DQCPIVIGLCHHNFHDHCIYRMLDTPTSKGLCPHCRQTFQL  165
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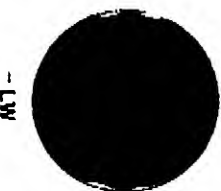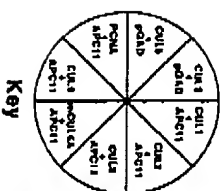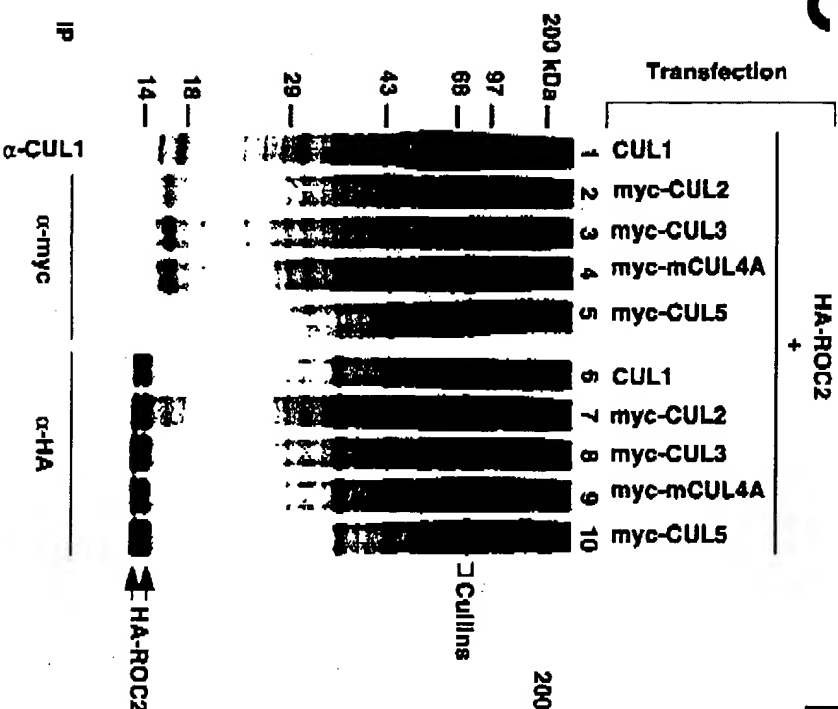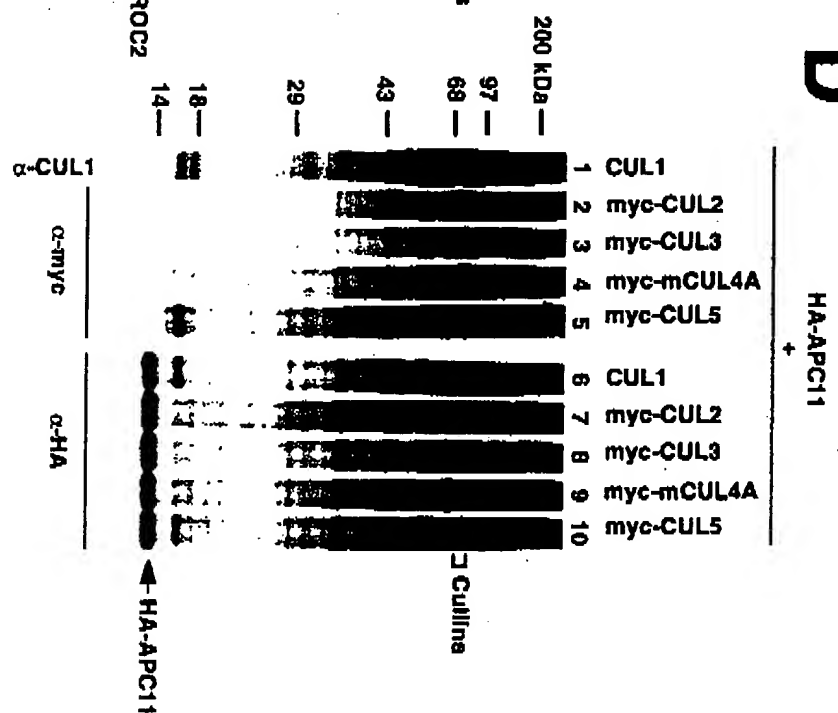
A

Transfection

1	CUL1			
2	myc-CUL2	HA-ROC1		
3	myc-CUL3	+		
4	myc-mCUL4A			
5	myc-CUL5			
6	myc-mCUL4A + pcDNA3			
7	CUL1			
8	myc-CUL2	HA-ROC1		
9	myc-CUL3	+		
10	myc-mCUL4A			
11	myc-CUL5			
12	myc-mCUL4A + pcDNA3			

200 kDa—
97—
68—
43—
29—
18—
14—

IP α-CUL1   α-myc   α-HA

←HA-ROC1

Cullins

B

Transfection

1	CUL1 + HA-ROC1		
2	CUL1 + pcDNA3		
3	myc-CUL2		
4	myc-CUL3	HA-ROC1	
5	myc-mCUL4A	+	
6	myc-CUL5		

IP α-HA

α-CUL1   α-myc

Cullins

Western α-HA

←HA-ROC1

Western

D

IP   α-CUL1   α-ROC1
pep   −   +   −   +
       1   2   3   4

CUL1 →

α-CUL1

Western

IP   α-CUL2   α-ROC1
pep   −   +   −   +
       5   6   7   8

CUL2 →

α-CUL2

Western

C

                    IVT   HeLa   Saos2
              ROC1 ROC1+CUL1
IP   α-ROC1
pep   −   −   +   −   +
       1   2   3   4   5   6

200 kDa —
                                    ⏋ Cullins
97 —
68 —

43 —

29 —

18 —
14 —   ← ROC1

A

Key

B

Key

C

Transfection

HA-ROC2 +

200 kDa —
97 —
68 —
43 —
29 —
18 —
14 —

IP   α-CUL1   α-myc   α-HA

1 CUL1
2 myc-CUL2
3 myc-CUL3
4 myc-mCUL4A
5 myc-CUL5
6 CUL1
7 myc-CUL2
8 myc-CUL3
9 myc-mCUL4A
10 myc-CUL5

Cullins

HA-ROC2

D

HA-APC11 +

200 kDa —
97 —
68 —
43 —
29 —
18 —
14 —

α-CUL1   α-myc   α-HA

1 CUL1
2 myc-CUL2
3 myc-CUL3
4 myc-mCUL4A
5 myc-CUL5
6 CUL1
7 myc-CUL2
8 myc-CUL3
9 myc-mCUL4A
10 myc-CUL5

Cullins

HA-APC11

A

PCNA + APC11	mAPC2 + APC11
mAPC2 + ROC2	mAPC2 + ROC1

Key   − LW   − LWH

B

Transfection

myc-mAPC2 + ... | myc-mAPC2 + ...

pcDNA3 | HA-ROC1 | HA-ROC2 | HA-APC11 | HA-APC11+ pcDNA3 | pcDNA3 | HA-ROC1 | HA-ROC2 | HA-APC11 | HA-APC11+ pcDNA3

1 2 3 4 5 6 7 8 9 10

200 kDa —

97 —

68 —

43 —

29 —

18 —

14 —

◄ myc-APC2

◄ HA-ROC1

◄ HA-ROC2

◄ HA-APC11

IP   α-HA   α-myc

HA-ROC1
+
pcDNA3

A

CUL1
+
pcDNA3

C

CUL1
+
HA-ROC1

E   F

CUL1
+
HA-APC11

H   I

myc-
mCUL4A
+
pcDNA3

K

myc-
mCUL4A
+
HA-ROC1

M   N

α-HA   α-CUL1
            or
            α-myc   Phase

Table 1. Subcellular Localization of Cullins and HA-ROC1

Transfection		Cytoplasmic	Nuclear + Cytoplasmic	Nuclear (%)
		Localization of Cullins		
CUL1	+ pcDNA3	92 ± 4	6 ± 4	2 ± 2
CUL1	+ HA-ROC1	21 ± 8	41 ± 5	38 ± 14
CUL1	+ HA-APC11	87 ± 5	9 ± 2	4 ± 3
myc-mCUL4A+	pcDNA3	91	6	3
myc-mCUL4A+	HA-ROC1	38	28	34
myc-CUL5	+ pcDNA3	47	44	9
myc-CUL5	+ HA-ROC1	0	9	91
		Localization of HA-ROC1		
HA-ROC1	+ pcDNA3	0 ± 0	9 ± 4	91 ± 4
HA-ROC1	+ CUL1	0 ± 1	28 ± 10	72 ± 10